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EFFECT OF COMPOSITION OF A GAS MIXTURE ON GROWTH OF BACTERIA ASSIMILATING GASEOUS HYDROCARBONS

Z. S. Smirnova

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EFFECT OF COMPOSITION OF A GAS MIXTURE ON GROWTH OF BACTERIA ASSIMILATING GASEOUS HYDROCARBONS

Z. S. Smirnova[1]

ABSTRACT: The results of studies of the effect of the composition of a gas mixture on the growth of bacteria oxidizing methane and propane are presented. It was found that the biomass concentration in the medium (all other conditions being equal) is directly proportional to the concentration of hydrocarbon and oxygen in the gaseous mixture. An oxygen concentration of up to 50% in a medium with methane and up to 55% in a medium with propane will not inhibit the growth of bacteria. /30*

The degree of utilization of the methane or propane for the construction of cell substance depends on the ratio of hydrocarbon and oxygen. Methane is most effectively utilized by bacteria at a volume ratio between O_2 and CH_4 equal to 1.5. Propane assimilation takes place most effectively at an O_2:C_3H_8 ratio equal to 3.

It is shown that carbon dioxide is required for growth of methane-oxidizing bacteria. The optimum CO_2 concentration is between 5 and 10%. A higher concentration of hydrocarbon has an inhibiting effect on the development of gas-oxidizing bacteria. A CO_2 concentration above 20% completely suppresses growth.

INTRODUCTION

Many papers have been devoted to a study of the composition of a gaseous mixture which is optimal for the development of gas-oxidizing bacteria, but there is no uniformity of opinion in regard to this question. Many authors are of the opinion that the hydrocarbon concentration does not have a significant influence on the growth of bacteria. Most of the disagreement hinges on the question of oxygen concentration.

Gaseous mixtures consisting of 10 to 20% propane and 75 to 85% air are usually employed for growing microorganisms in a propane atmosphere, i.e., the

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[1] Institute of Biochemistry and Physiology of Microorganisms, Academy of Sciences of the USSR.

* Numbers in the margin indicate foreign pagination.

oxygen concentration does not exceed 15 to 17% (Dostalek, 1954; Bogdanova, 1961; Davis, 1964). Those authors who used mixtures of gas and air for cultivating methane-oxidizing bacteria consider a methane-air ratio of 1:2 to be optimal for growth, i.e., a mixture which contains a maximum of 15% oxygen (Soehngen, 1906; Muenz, 1915; Dworkin, Foster, 1956). In their opinion, a higher concentration of oxygen is destructive. At the same time, however, many investigators who have used a mixture of methane and pure oxygen for cultivation of bacteria (Hutton, ZoBell, 1949; Johnson, Templ, 1962; Brown et al., 1964; Wolnak et al., 1967) note optimum growth with 30 to 45% oxygen in the atmosphere.

Data are available on the stimulating effect of low concentrations of carbon dioxides (5 to 10%) on the growth of bacteria which oxidize methane or propane (Hutton, ZoBell, 1949; Johnson, Templ, 1962; Wolnak et al., 1967; Dostalek, 1954). The question of the influence of higher concentrations of carbon dioxide on the vital activity of gas-oxidizing bacteria is little discussed in the literature.

The purpose of the present paper is to study of the influence of the ratio of hydrocarbon and air as well as an increased concentration of oxygen and carbon dioxide in a gaseous mixture on the growth of gas-oxidizing bacteria developing under the conditions in a closed system.

As the objects of the investigation, we used cultures of bacteria isolated /31
in an atmosphere of methane or propane from soil samples collected in oil-bearing regions of the USSR (80-M, 9-K; 1-A; 2-A; 3-A and 5-A), from soil in the Moscow Oblast', contaminated by petroleum products (39-M and 5-L), from the sapropel of a lake in Mytishi (5-S) and from sludge taken from Lake Tambukan (3-S).

As the nutrient medium in these experiments, we used the Bushnell-Haas mineral medium. In isolated cases we used rider medium or medium number 8. The composition of the media is given in a previous paper (Smirnova, 1968).

The effect of the ratio of hydrocarbon and air on the growth of gas--oxidizing bacteria was studied in a cumulative culture of 5-L bacteria and three pure cultures of mycobacteria (strains 80-M, 39-M and 9-K).

The microorganisms were grown with agitation on a rocking device (180 cycles per minute) in flasks with a capacity of 2.6 liters (the 80-M strain was cultured in a 1-liter flask), filled with a gaseous mixture. The cultivation time was 6 days. We tested gaseous mixtures with methane and air ratios of 1:2, 1:6, 1:8.5 and mixtures of propane and air in the ratios of 1:1, 1:2, 1:5, 1:9, 1:12, 1:14 and 1:24. The concentration of oxygen in the gaseous mixtures of methane and air was 1:8.5 and in those of propane and air (1:24) it was about 18% (by calculation).

The effect of higher concentrations of oxygen in a gaseous mixture on the growth of bacteria was studied using the example of an active culture (9-K) utilizing propane, and a culture (1-A) which developed intensively in a methane atmosphere. The microorganisms were grown by the deep method with a pH of 7.2 for the medium (culture 9-K) and at pH = 6.7 (culture 1-A) in gas mixtures containing some 15 to 54% oxygen. The duration of the experiments did not exceed 48 hours.

The effect of carbon dioxide on the growth of methane-oxidizing bacteria was studied in 6 cultures by growing them in a gas mixture consisting of 51.5% CH_4 and 48.5% O_2 (O_2/CH_4 = 1.3) with and without addition of 5% CO_2.

Cultivation took place during agitation in the flasks with baffles (capacity equals 1 liter) under conditions involving regulation of the pH of the medium and replacement of the gas mixture every 48 hours. The pH was corrected by a 10% solution of NaOH. The experiment lasted 7 days. The incubation temperature in all the experiments was 29 to 30°. The growth of the microorganisms was determined nephelometrically using the FEKN-58 apparatus (1 milliliter cuvette) and on the basis of the weight of the dry biomass on number 3 membrane filters. The analyses of the gas mixtures were performed on a VTI-2 gas analyzer. When collecting gas samples for analysis at the beginning and end of the experiment, the temperature and atmospheric pressure were measured.

The volumes of the individual components of the gaseous mixture were calculated according to the formula

$$V_{1,2,3} = \frac{Ve'' \cdot P \cdot 273 \cdot V'x}{760 \cdot T \cdot V'e},$$

where $V_{1,2,3}$ is the volume of the components corrected for normal conditions; Ve'' is the volume of the gas in the flasks; P is the atmospheric pressure in millimeters of mercury Hg, Ve' is the volume of gas collected for analysis; $\hat{X}_x$ is the percentage of the component in volume Ve''; T is $(273° + t°)$, $t°$ is the temperature at the time of measurement.

RESULTS AND EVALUATION

Hydrocarbon and air ratio. The results of the experiments in growing microorganisms in gas mixtures with different ratios of hydrocarbon and air, shown in Table 1, indicate that the ratio of methane and propane to air has a significant influence on the technological yield (V_t), i.e. the yield of biomass relative to the weight of hydrocarbon consumed in the experiment.

It follows from Table 1 that as the relative content of oxygen in the gaseous mixture increases, the output of biomass increases as well. Under the conditions in which the experiments were performed, the highest technological yield for a medium with methane ($V_t \doteq 65\%$) was obtained when growing a culture of 5-L bacteria in a gaseous mixture consisting of 1 volume of methane and 8.5 volumes of air (upon conversion for oxygen, the ratio of O_2/CH_4 in this mixture is 1.7). /32

When growing microorganisms on a medium with propane, the highest yield of biomass ($V_t = 89\%$) was obtained with a propane and air ratio in the gaseous mixture of 1:14 ($O_2/C_3H_8 = 2.8$). With a lower or higher relative content of air (propane:air = 1:12 or 1:24), the biomass yield per weight of collected propane decreases.

With the same ratio of hydrocarbon and air, the concentration of the synthesized biomass is directly proportional to the quantity of hydrocarbon in the gaseous atmosphere (Table 2).

Oxygen. The concentration of oxygen in gas-air mixtures used in the experiments described above, as we have already mentioned, did not exceed 18%.

TABLE 1: INFLUENCE OF THE RATIO OF HYDROCARBON AND AIR IN A GASEOUS MIXTURE ON THE BIOMASS YIELD DURING THE UTILIZATION OF METHANE AND PROPANE BY MICROORGANISMS

Microorganisms	Medium	Source	Hydrocarbon in Gaseous Mixture	Hydrocarbon: Air (volume for volume)	Duration of Experiment, Days	Biomass, mg (dry weight)	(v_t), %
1	2	3	4	5	6	7	8
5-л	Rider	CH_4	805	1:2	6	79	14,0
			420	1:6		93	31,6
			225	1:8,5		102	65,0
M. Flavum, 9-K	No. 8	C_3H_8	1200	1:1		212	8,8
			800	1:2	3	170	10,6
			400	1:5		237	29,6
			200	1:12		257	64,2
M. Lacticolum, 39-M	Bushell-Haas	C_3H_8	1200	1:1	6	109	4,5
			400	1:5		170	22,5
			325	1:14		580	89,0
M. Lacticolum, 80-M	Bushell-Haas	C_3H_8	100	1:2	6	15	7,5
			100	1:5		38	19,0
			100	1:9		59	29,5
			100	1:24		98	49,0

TABLE 2: INFLUENCE OF THE QUANTITY OF HYDROCARBON IN A GAS MIXTURE ON THE YIELD OF BIOMASS.

It was interesting to determine how the growth of gas-oxidizing bacteria is affected by higher concentrations of oxygen. Cultivation of microorganisms in mixtures of hydrocarbon and air with addition of pure oxygen has shown (Table 3, 4) that the concentration of oxygen in a gaseous mixture above 18%, all the way to 50% in a medium with methane and up to 55% in a medium with propane, does not have an inhibiting effect on the growth of bacteria. /33

TABLE 3: EFFECT OF HYDROCARBON AND OXYGEN CONCENTRATION ON THE GROWTH OF A CULTURE OF METHANE-OXIDIZING BACTERIA (1-A)

Composition of Gas Mixture, Volume %			O_2/CH_4	Methane Used, mg	Biomass Formed After 36 Hours, mg	v_{φ}, %
Oxygen	Methane	Nitrogen				
15,6	29,8	54,6	0,5	33,0	6,6	20
22,5	37,5	40,0	0,6	42,4	10,6	25
19,0	20,5	60,5	0,9	23,3	11,6	50
49,7	50,3	0,0	0,9	102,4	56,7	54
24,0	20,0	56,0	1,2	10,2	9,0	90
28,5	19,0	52,5	1,5	12,7	12,9	100
26,0	12,5	61,5	2,0	40,5	14,2	35
26,7	11,3	62,0	2,3	40,9	12,0	30

TABLE 4: GROWTH OF *M. Flavum* (9-K) IN A PROPANE ATMOSPHERE AS A FUNCTION OF THE CONCENTRATION OF OXYGEN IN THE GASEOUS ATMOSPHERE AND ITS RATIO TO THE HYDROCARBON

Propane M_9			O_2/C_3H_8	Пропан, мг	Biomass Formed After 72 Hours, mg	v_t, %
Oxygen	Propane	Nitrogen				
26,8	50,0	23,2	0,5	320	24	7,5
41,6	57,0	1,4	0,7	303	30	9,2
53,3	47,0	0	1,1	259	35	13,7
54,3	45,7	0	1,2	252	50	19,8
37,6	20,7	41,7	1,8	114	56	49,1

It follows from the data in Table 3 that with all other conditions being equal, the quantity of biomass synthesized by the microorganisms depends both on the concentration of hydrocarbon and the oxygen concentration in the gaseous mixture. However, the degree of utilization of the hydrocarbon for construction of cell substance, as indicated by Table 3 and 4, does not depend on the concentration of hydrocarbon and oxygen but on their ratio in the gaseous mixture.

Carbon dioxide. With 5% carbon dioxide in the gaseous atmosphere, there is a considerable reduction in the duration of the lag phase (the phase during which growth is inhibited). The results which were obtained support the findings of other authors regarding the stimulating effect of small concentrations of carbon dioxide on the growth of bacteria which oxidized methane (Table 5).

It is clear from Table 5 that carbon dioxide exerts influence not only on the growth rate but also on the final yield of cells. Thus, when growing 6 cultures of bacteria in a gas mixture with addition of 5% CO_2, the quantity of biomass increased by 2 1/2 times on the average in comparison to the controls. It should be pointed out that the utilization of the gas mixture (as converted for 1 milligram of biomass) is about 2 1/2 times less.

We can gain some idea of the effect of a higher concentration of carbon dioxide on the growth of bacteria which oxidized methane from the results which were obtained when culture 1-A was grown in a fermenter with a capacity of 3 liters with a constant pH of the medium equal to 6.8--7.0. The volume of medium was 1 liter. The mediume was agitated with a bladed stirrer at the rate of 1200 rpm. The fermenter was connected to a gasometer with a volume of 10 liters, filled with a gas mixture of the same composition as was in the fermenter (%): /34

$$CH_4\text{--}40;\ O_2\text{--}53;\ CO_2\text{--}0.5;\ N_2\text{--}6.5.$$

As a result of the rarefaction which took place during the development of the bacteria in the fermenter, the gas mixture was automatically drawn into it from the gasometer. The rate of utilization of the gaseous mixture could be estimated from the level of the fluid in the gasometer. At the beginning of the experiment and every 12 hours thereafter, samples of gas and culture fluid were collected from the fermenter to determine the state of the gas mixture and the optical density of the cell suspension. The experiment lasted 84 hours.

TABLE 5: INFLUENCE OF CARBON DIOXIDE ON THE GROWTH OF METHANE-OXIDIZING BACTERIA

Microorganisms	Duration of Lag Phase, Hours		Biomass on the 7th Day, mg per liter (dry weight)		Utilization of Gaseous Mixture, Milliliters per mg of Biomass	
	without CO_2	5% CO_2	without CO_2	5% CO_2	without CO_2	5% CO_2
3-C	96	3	0,2	1,3	7,0	1,0
5-C	96	12	0,3	1,3	1,0	1,2
1-A	60	8	0,6	1,5	7,0	2,5
2-A	72	3	0,8	1,2	9,8	2,5
3-A	120	12	0,2	0,7	8,1	4,3
5-A	75	2	0,8	1,4	6,0	5,7

TABLE 6: RELATIONSHIP BETWEEN CONCENTRATION OF HYDROCARBON FORMED, GROWTH AND RESPIRATORY ACTIVITY OF A CULTURE OF METHANE-OXIDIZING BACTERIA (1-A)

Culturing Time, Hours	Composition of Gas Mixture, Volume %				O_2/CH_4	Specific Growth Rate (microns)	$\frac{CO_2}{O_2}$	Units (of optical density)
	CH_4	O_2	CO_2	N_2				
0	40	53	0,5	6,5	1,3	—	—	0,01
24	—	35	5,5	—	—	0,09	0,3	0,08
48	—	26	13,4	—	—	0,03	1,0	0,20
72	—	24	19,7	—	—	0,02	1,6	0,32
84	18	23	34,8	24,2	1,3	0,01	3,0	0,35

The results of this experiment, shown in Table 6 and in Figure 1, indicate that the process of growth of the microorganism is accompanied by an intensive absorption of the gaseous mixture composed of methane and oxygen, development of biomass and an increase in the concentration of the hydrocarbon which is formed. The highest specific growth rate ($\mu = 0.09$) and respiratory coefficient close to theoretical $\frac{CO_2}{O_2} = 0.3$, was observed 24 hours later, when the hydrocarbon concentration reached 5.5% (Table 6). The most intensive utilization of the gaseous mixture took place at a carbon dioxide concentration of 8 to 13%, but the specific growth rate and the respiratory activity, as we can see from Table 6, decreased at this time. With a further increase of the concentration of carbon dioxide in the fermenter, there was a decline in the rate of absorption of the gas mixture and the specific rate of growth of the microorganism.

The sharp increase in the respiratory coefficient indicates that as the /35 concentration of CO_2 in the gaseous mixture increases, the intensity of respiration decreases. It is apparent from Table 6 and Figure 1 that a CO_2 concentration above 15% will suffice to considerably inhibit the growth and respiratory activity of methane-oxidizing bacteria. With a hydrocarbon concentration above 20%, growth ceases completely. Inasmuch as the growth of the 1-A culture took place under conditions in which the pH of the medium was maintained at a given level and the residual concentration of methane and oxygen in the gaseous mixture was equal to 18 and 23%, respectively, the factors indicated could not have limited the growth of the bacteria. It may be that the ceasation of the growth of the bacteria was caused by the effect of a high concentration of carbon dioxide.

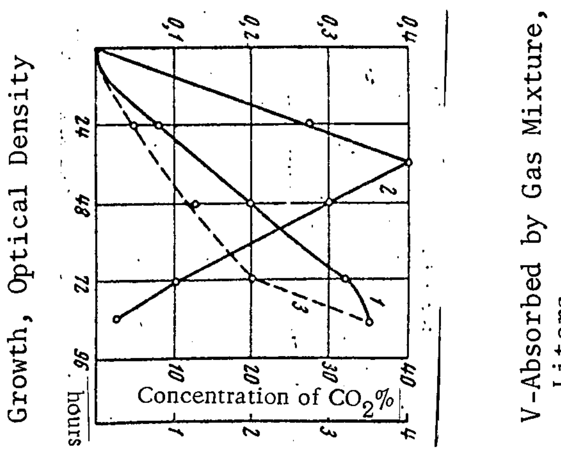

Figure 1. Growth (1), Rate of Utilization of a Gaseous Mixture (2) and Formation of Hydrocarbon During Oxidation of Methane by Culture 1-A.

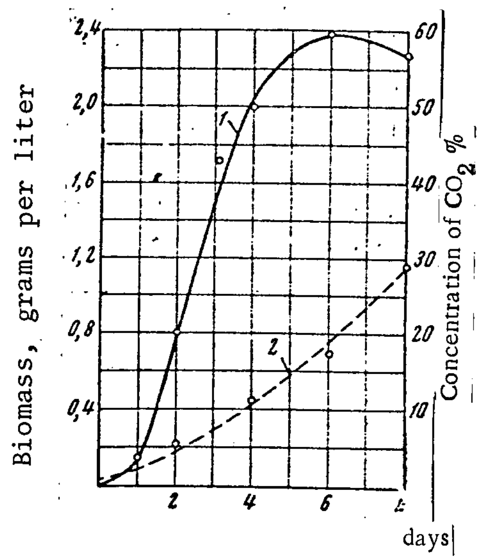

Figure 2. Growth of a Culture of Mycobacteria 9-K (1) and Formation of Carbon Dioxide (2) During Oxidation of Propane.

A similar situation was observed with the decultivation of *M. flavum* 9-K in an atmosphere of propane and oxygen. The culture was grown in a flask with agitation, using mineral medium number 8 with a pH of approximately 7 for the medium. The ratio of the object O_2/C_3H_8 in the gaseous mixture was 2.4.

Results of the experiment, shown in Table 7 and Figure 2, indicate that the concentration of hydrocarbon produced when propane is oxidized inhibits the growth of the 9-K strain when it rises above 7%. With a concentration of carbon dioxide equal to 18%, the growth ceases although the quantity of propane and oxygen in the gaseous mixture remain sufficiently high. Hence, the results of the studies which were performed indicate that when microorganisms are cultivated on medium with gaseous hydrocarbons the composition of the gaseous mixture is one of the most important factors affecting growth.

The data obtained indicated that the yield of biomass with other conditions being equal depends on the concentration of hydrocarbon and oxygen. Increasing the quantity of oxygen in the gaseous mixture to 50% will not inhibit the growth of the bacteria which oxidized methane or propane. On the contrary, replacement of the air by pure oxygen will make it possible to increase the concentration of hydrocarbon to 50% and thereby considerably increase the yield of biomass per unit volume of medium.

Poor growth or absence of growth by the bacteria in an atmosphere of /36
methane or propane with an oxygen concentration in the gas-air mixture above 15 to 17%, obtained in the experiments of certain authors, may be explained most likely by the limiting affect of a very low concentration of hydrocarbon.

The data which we obtained indicate that the degree of utilization of hydrocarbon for construction needs depends on the ratio of hydrocarbon and oxygen in the gaseous mixture.

Under the conditions existing in the experiments which were performed, the highest uield of biomass per methane utilized (V_f = 100%) or per methane added (V_t = 65%) where obtained when the methane-oxidizing bacteria were grown in gas mixtures with a ratio of O_2/CH_4 equal to 1.5-1.7. On a medium with propane, the highest yield of biomass relative to the weight of hydrocarbon used in the experiment (V_t = 89%) was observed when growing mycobacteria in a mixture of propane and air of 1:14 where O_2/C_3H_8 approximately 3.

TABLE 7: INFLUENCE OF CARBON DIOXIDE ACCUMULATED IN A CLOSED SYSTEM ON THE GROWTH OF A CULTURE OF *M. Flavum* 9-K DURING ITS OXIDATION OF PROPANE

Duration of Experiment, Days	Composition of Gas Mixture, Volume %			O_2CH_4	Biomass, grams per Liter (dry weight)
	C_3H_8	O_2	CO_2		
0	29,1	69,9	1,0	2,4	0,05
1	—	—	—	—	0,1
2	—	—	5,0	—	0,8
3	—	—	—	—	1,7
4	—	—	10,1	—	2,0
5	—	—	—	—	—
6	—	—	17,9	—	2,4
7	—	—	—	—	—
8	22,5	16,5	29,5	0,73	2,3

It was found that carbon dioxide in a concentration up to 10% will stimulate the growth of methane-oxidizing bacteria; when there is 5% CO_2 in the gas mixture, the growth rate increases and the gas mixture is utilized more economically.

The data obtained indicate that hydrocarbon participates in the synthesis of cell substance (heterotrophic assimilation of hydrocarbon takes place) and therefore its presence in the gas mixture is apparently necessary.

The results of the experiments which were performed agree with the data in the literature which indicate an increased ability of methane-oxidizing bacteria to perform heterotrophic assimilation of carbon dioxide (Quayle, 1963; Sorokin, 1961; Johnson, Templ, 1962).

However, as the studies which were performed indicate, a higher concentration of carbon dioxide (more than 10%), stored in a closed system with oxidation of a gaseous hydrocarbons, inhibits the growth of bacteria. Consequently, CO_2 as a product of metabolism is one of the factors which limit the growth of gas-oxidizing bacteria. The toxic effect of high concentrations of CO_2 (greater than 10%) on the growth of other microorganisms has been noted by many investigators (Baranova, 1953).

An analysis of the results obtained indicates that the optimum gas mixture for growing methane-oxidizing bacteria evidently is a mixture composed of 50% methane, 45% oxygen and 5% CO_2.

When cultivating in a closed system, it is necessary to insure cirulation of the gaseous mixture with removal of excess carbonic acid which is formed in the course of oxidation of the hydrocarbon.

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